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THERMAL STABILITY OF LYSOZYME AND MYOGLOBIN IN THE PRESENCE OF ANIONIC SURFACTANTS

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The interactions of lysozyme and myoglobin with anionic surfactants (hydrogenated and fluorinated), at surfactant concentrations below the critical micelle concentration, in aqueous solution were studied using spectroscopic techniques. The temperature conformational transition of globular proteins by anionic surfactants was analysed as a function of denaturant concentration through absorbance measurements at 280 nm. Changes in absorbance of protein-surfactant system with temperature were used to determine the unfolding thermodynamics parameters, melting temperature, T_m, enthalpy, ΔH_m, entropy, ΔS_m and the heat capacity change, ΔC_p, between the native and denatured states.

Keywords: *lysozyme, myoglobin, thermal denaturation, thermodynamics, UV absorbance*

Introduction

The application of surfactants in the field of biochemistry has given importance to studies of the nature of the interaction between protein and surface active agents in biological phenomena such as biological membranes [1] and protein solubilization. It has also been suggested that surfactant-protein systems can be used as a model for biological membranes [2, 3].

Proteins and ionic surfactants show the property of having both charged groups and hydrophobic portions [4]. This implies that the interactions between surfactants and proteins are complex processes, involving different types of intermolecular forces. Therefore, the ionic head groups of surfactants may bind to oppositely charged groups on the protein surface by electrostatic forces, whereas non-polar tail groups of surfactants may bind to no-polar sites on the protein surface through hydrophobic forces [5].

The widespread use of the anionic surfactants has stimulated interest in the nature of the interactions between those surfactants and globular proteins. Depending on the surfactant concentration, the protein conformation will adopt a specific structure. The use of anionic surfactants like sodium alkyl carboxylates at low concentrations usually induces the compaction of protein (folding), nevertheless at moderate concentrations below the critical micelle concentration (*cmc*) are a potent denaturant for protein solution [6, 7].

The interaction of sodium alkyl carboxylates with lysozyme and myoglobin has been studied by UV spectroscopic methods [8–10], for whom, we have done the analysis of thermal unfolding curves.

Lysozyme is a small protein, molecular mass 14.3 kDa, of 129 amino acids, containing 18 cationic amino acid residues and 12 anionic residues. Its structure is stabilized by four disulfide bridges and the interior of the protein is almost hydrophobic while the surface is mostly polar. The isoelectric point is pI~11.0 and the protein is therefore positively charged in an aqueous solution.

Myoglobin is a single-chain protein, molecular mass 16.7 kDa, of 153 amino acids, containing a heme group in the center. Its secondary structure is unusual in that it contains a very high proportion (75%) of α-helical secondary structure. The isoelectric point is pI~7.3.

Work reported includes the analyse of the thermal denaturation curves for single protein in aqueous solution and for protein plus surfactants at different concentrations, determination of temperature dependence of ΔG [11], melting temperature (T_m) and others thermodynamics parameters at T_m like ΔH_m, ΔS_m and ΔC_p.

Experimental

Materials

Sodium octanoate (C_8HONa) and sodium perfluorooctanoate (C_8FONa) of at least 97% purity were obtained from Lancaster Synthesis Ltd. Sodium dodecanoate ($C_{12}HONa$) with purity greater than 99%, was obtained from Sigma Chemical Co.

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Lysozyme (from chicken egg white, product L-6876, Sigma Chemical Company) and myoglobin (from equine skeletal muscle, product M-0630, Sigma Chemical Company) were used without further purification. All solutions were made in distilled water. Protein concentration (0.125 g dm^{-3}) was kept constant in all experiments. Stock solutions of defined protein concentration were prepared by dissolving a certain amount of freeze-dried protein in an appropriate buffer volume.

Methods

Difference spectra were measured using a Beckman spectrophotometer (model DU 640), with six microcuvettes, operating in the UV-Visible region, from 190 to 1100 nm of the electromagnetic spectrum wavelength, with a full scale expansion of 0.2 absorbance units. All measurements were made using lysozyme and myoglobin solutions with a concentration of 0.125 g dm^{-3} in carefully matched quartz cuvettes (50 μL capacity) in the wavelength range 200–500 nm. For absorbance difference spectra, five of the six microcuvettes were filled with protein plus surfactant solutions. The first microcuvette contained only protein in the corresponding medium and was used as a blank reference. The microcuvettes were filled and placed in the same position for all the tests. Measurements were made after lysozyme, myoglobin and surfactant had been incubated for over 30 min, time during which the difference spectra did not change. For absorbance measurements with changing temperature, a Beckman (DU Series) temperature controller was used, within a ±0.01 K, following the Peltier methods of controlling temperature, in the range 288 to 373 K.

Results and discussion

From the thermodynamic point of view, the denaturation process can be described as a transition between two macroscopic states [12, 13], that is, from the native state (N) to a denaturated state (D).

$$N \Leftrightarrow D \tag{1}$$

Stability of a globular protein is usually quantified in the Gibbs energy values, since ΔG is the work required for disruption of the native protein structure. For that reason, the difference in Gibbs energy at a given temperature can be expressed by the Gibbs–Helmholtz equation:

$$\Delta G(T)=\Delta H_m(1-T/T_m)-\Delta C_p[T_m-T+T\ln(T/T_m)] \tag{2}$$

where T_m refers to the temperature at the midpoint of the transition ($T_m=T$ at $\Delta G=0$), ΔH_m is the enthalpy of denaturation at the T_m and ΔC_p is the heat capacity change between the folded and unfolded conformations.

The evaluation of thermodynamic parameters, obtained from spectroscopic techniques, is based on the equilibrium constant K for a transition between the native state and the denaturated state. The equilibrium constant was deduced from the equation:

$$K=\frac{[\text{unfolded}]}{[\text{native}]} \tag{3}$$

or as a function of spectroscopic parameters [14–16]:

$$K=\frac{(A_N-A_0)}{(A_0-A_D)} \tag{4}$$

where A_N is the absorbance of the pure native state, A_D is the corresponding absorbance of the pure denatured state, and A_0 is the observed absorbance at any temperature in the transition zone.

To avoid large errors at the estimation of the thermodynamic parameters [17], we have used all the experimental data points obtained and fitted them in the equations in the following manner:

$$A_0=\frac{(A_N+KA_D)}{(1+K)} \tag{5}$$

On the other hand, the equilibrium constant can be expressed by a Gibbs energy function:

$$K=e^{-\Delta G/RT} \tag{6}$$

where R is the gas constant and T is the temperature in K. Substituting the value of K in Eq. (5) we get:

$$A_0=\frac{A_N+A_D e^{-\Delta G/RT}}{1+e^{-\Delta G/RT}} \tag{7}$$

Finally, substituting the $\Delta G(T)$ expression for Eq. (2), we get:

$$A_0=\frac{A_N+A_D\exp\left[-\frac{1}{R}\left\{\Delta H_m\left(\frac{1}{T}-\frac{1}{T_m}\right)\right\}-\Delta C_p\left[\frac{T_m}{T}-1+\ln\left(\frac{T}{T_m}\right)\right]\right]}{1+\exp\left[-\frac{1}{R}\left\{\Delta H_m\left(\frac{1}{T}-\frac{1}{T_m}\right)\right\}-\Delta C_p\left[\frac{T_m}{T}-1+\ln\left(\frac{T}{T_m}\right)\right]\right]} \tag{8}$$

Since A_N and A_D have been found to be linear functions of temperature, they can be written as:

$$A_N=a_N+m_N T \tag{9}$$

$$A_D=a_D+m_D T \tag{10}$$

The difference in free energy between folded and unfolded conformations, ΔG, depends mainly on the first term at temperatures near T_m, meanwhile the second term becomes important at temperatures below T_m [10].

The absorbance at 280 nm as a function of temperature is shown in (Fig. 1) for different globular proteins. After denaturation, the protein solution was cooled to room temperature to measure the absorption and to compare with that of the unheated sample, this agreement is taken as a measure of reversibility of the heat-induced denaturation. In all the cases, the thermal transitions were reversible and hence amenable to thermodynamic analysis.

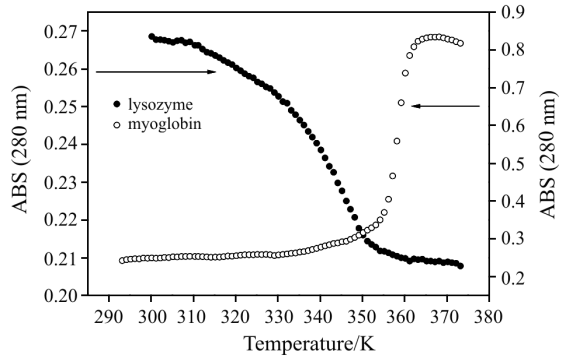

Fig. 1 Thermal unfolding curves for lysozyme and myoglobin (0.125 g dm^{-3}) in aqueous media

Table 1 Parameters characterising the thermal unfolding of lysozyme-surfactant system

[M]/ mmol L^{-1}	T_m/ K	ΔH_m/ kJ mol^{-1}	ΔS_m/ kJ mol^{-1} K^{-1}	ΔC_p/ kJ mol^{-1} K^{-1}
		C_8FONa		
0.00	340.6	309.42	0.91	7.32
0.01	344.7	402.60	1.17	15.64
0.02	344.7	438.99	1.27	15.82
0.03	346.0	417.21	1.20	18.07
0.04	345.8	404.96	1.17	12.78
0.05	345.1	490.51	1.42	17.55
0.06	345.4	540.16	1.56	19.20
0.07	346.2	424.27	1.22	17.47
0.08	345.2	403.56	1.17	16.77
0.10	345.8	230.69	0.67	7.18
		C_8HONa		
0.2	348.1	335.05	0.96	8.90
0.4	349.7	327.71	0.94	8.29
0.5	347.5	397.40	1.14	8.41
0.7	351.9	359.61	1.02	10.52
0.8	354.1	184.37	0.52	16.31
1.0	353.1	502.52	1.42	17.78
1.2	353.7	371.60	1.05	6.38
1.4	357.6	371.12	1.04	10.11
1.5	354.4	379.88	1.08	9.85
1.6	358.2	298.51	0.83	11.37
		C_{12}HONa		
0.05	347.0	391.63	1.13	15.90
0.10	348.9	372.96	1.07	17.73
0.20	349.4	346.34	0.99	20.63
0.25	350.7	295.08	0.84	10.37
0.30	348.4	401.35	1.15	13.39
0.35	340.9	330.77	0.97	16.45
0.40	336.3	276.72	0.82	14.60

T_m values were found to be within ±0.5 K; uncertainties in ΔH_m, ΔS_m and ΔC_p were within ±10%

The data show that there is a transition region over which the absorbance changes with temperature. Analyzing these curves, T_m values of 67.46 and 85.03°C for lysozyme and myoglobin have been obtained respectively. Similar curves were found when we analysed the absorbance changes of protein-surfactant system. For all samples, the same protein concentration 0.125 g dm^{-3}, and the surfactant concentration range was chosen low enough to guarantee that the binding sites are unsaturated (Tables 1 and 2). Therefore, in mixed systems alkyl carboxylates-proteins, at very low surfactant concentrations, electrostatic is the dominant interaction rather than hydrophobic ones.

Figures 2–4 show the melting temperature values (T_m) *vs*. surfactant concentration. It is worthy analyzing a comparative effect between the same surfactant and the two proteins. Figure 2 shows that the interaction protein-C_8HONa results in an increase of T_m. For that reason, we could say that in the con-

Table 2 Parameters characterising the thermal unfolding of myoglobin-surfactant system

[M]/ mmol L^{-1}	T_m/ K	ΔH_m/ kJ mol^{-1}	ΔS_m/ kJ mol^{-1} K^{-1}	ΔC_p/ kJ mol^{-1} K^{-1}
		C_8FONa		
0.00	358.18	668.67	1.87	17.62
0.10	356.42	766.94	2.15	26.62
0.20	355.83	658.23	1.85	21.51
0.30	356.41	934.57	2.62	43.60
0.40	355.81	888.24	2.50	46.49
0.50	356.43	797.39	2.23	28.51
0.55	356.14	667.43	1.87	38.13
0.60	355.74	878.43	2.47	40.66
0.70	355.86	630.94	1.77	54.58
0.80	356.48	321.95	0.90	35.09
0.90	356.50	250.32	0.70	31.90
1.00	359.38	264.63	0.74	29.32
		C_8HONa		
1	358.72	1157.90	3.23	24.40
2	359.66	1016.64	2.83	26.85
3	359.75	915.92	2.55	34.62
4	360.75	564.73	1.57	14.24
5	360.74	871.52	2.42	23.78
7	361.29	670.68	1.86	12.93
		C_{12}HONa		
0.03	358.78	944.86	2.63	37.97
0.05	358.87	972.81	2.71	55.75
0.07	359.89	829.35	2.30	62.91
0.10	360.79	716.84	1.99	27.93
0.15	360.49	862.95	2.39	18.75
0.17	359.32	839.22	2.34	6.23
0.20	358.65	310.29	0.87	10.35

T_m values were found to be within ±0.5 K; the uncertainties in ΔH_m, ΔS_m and ΔC_p were within ±10%

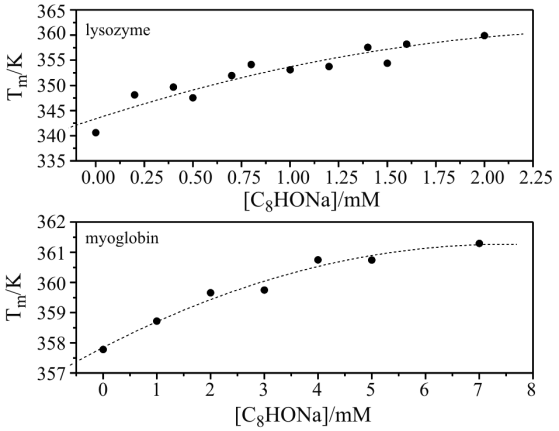

Fig. 2 T_m of lysozyme and myoglobin as a function of sodium octanoate concentrations

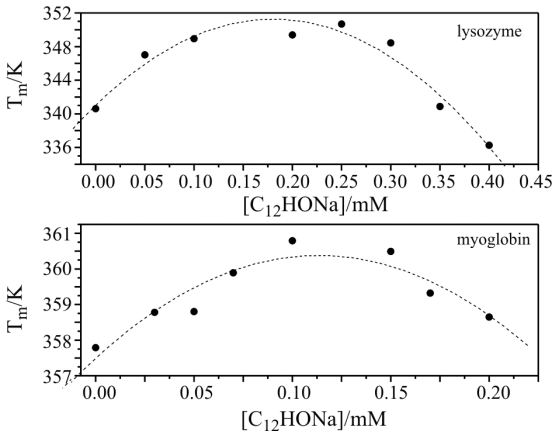

Fig. 3 T_m of lysozyme and myoglobin as a function of sodium dodecanoate concentrations

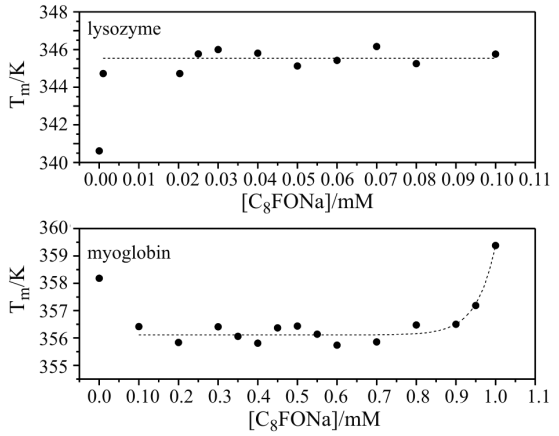

Fig. 4 T_m of lysozyme and myoglobin as a function of sodium perfluorooctanoate concentrations

centration studied range, C_8HONa increases the protein stability. This effect is higher for lysozyme where ΔT_m=20°C whereas for myoglobin ΔT_m=4°C. In general, the presence of anionic surfactants at very low concentrations induces the compaction of globular proteins, but this effect is increased in the case of surfactants with small chain length [6]. It is worth mentioning that C_{12}HONa concentrations over 0.3 mM results in the precipitation of the system. The formation of this precipitate is as a result of the neutralization of the positively charged protein (pI=11) by this anionic surfactant [18] in aqueous solution. On the other hand, the isoelectric point of myoglobin in aqueous solutions is pI=7.3, this means that in our systems the net charge of the protein is close to zero. Therefore, the binding protein-surfactant system involves a hydrophobic interaction due to saturated ionic sites.

In relation to the effect of fluorinated surfactant, we have obtained that this surfactant produces a smaller protective effect in the protein structure than the other surfactants. The reason for this behaviour could be linked with the outstanding hydrophobic character of this type of surfactants. On the other hand, from Fig. 4, it quite clear that C_8FONa stabilize lysozyme and destabilize myoglobin. Probably the electrostatic interaction between surfactant and protein might be dominant in low concentration of the surfactant and the hydrophobic interaction might be dominant in the high concentration. In low concentration, the electric shielding of protein surface by surfactant would stabilize the protein. The effect for lysozyme may be larger than that for myoglobin because lysozyme has many positive charges in water. In high concentration, the hydrophobic interaction between surfactant and protein would influence the stability of protein at both states (native and denatured states). Denatured state has usually larger accessible surface area and hydrophobic surface than the native state, indicating that the denatured state can interact more surfactant molecules than the native state. Therefore the denatured state would be stabilized by surfactant compared with the native state. (In other word, the native state would be relatively destabilized by stabilizing of the denatured state.) In fact, as shown in Fig. 3, C_{12}HONa destabilized the protein at high concentration (but lower than C_8HONa) probably because C_{12}HONa has more effective hydrophobic interaction than C_8HONa due to having the long chain length.

Regarding thermodynamics parameters, positive values of ΔH_m have been obtained, this is, these processes are endothermic. As entropy is a measure of disorder in the system, we have obtained that entropy of denaturation (ΔS_m) is positive; therefore the denatured state is more disordered than the native state. This behaviour is related with the fact that at room temperature, the entropies of hydration of both non-polar and polar residues are negative indicating that both create order in the aqueous environment. However these entropies differ with respect to how they change with increasing temperature. The entropy of hydration of non-polar residues increases through

zero with increasing temperature, indicating that they are less able to order the water at higher temperatures and may, indeed, contribute to its disorder by interfering with the extent of the hydrogen-bonded network and allowing an easier molecular rotation of water. Also, there is an entropy gain from the greater freedom of the non-polar groups when the protein is unfolded. In contrast, the entropy of hydration of polar groups decreases, becoming more negative with increasing temperature, as they are able to create ordered hydration shells even from the more disordered water that exists at higher temperatures. Finally, to our knowledge there is no DSC data for these same systems, however they are in same order that those found for similar systems [13].

Conclusions

Interactions between protein and C_8HONa (surfactant with a small chain length) lead to protein folding, which probably is related to protective effect of surfactant in protein by means of binding bridges between non-polar sites of protein and polar sites by hydrophobic and electrostatic interactions respectively. This protective effect is higher in the case of lysozyme. Positive values obtained for ΔS_m highlight an increase in the disorder of the system. On the other hand, enthalpy positive values reveal that these kinds of reactions are mainly endothermic.

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